Subterranean Biology 10: 17-24, 2012 (2013)
doi: 10.3897/subtbiol.10.3915

Shape variation of *Aegla schmitti* (Crustacea, Decapoda, Aeglidae) associated to superficial and subterranean stream reaches

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ABSTRACT
The species *Aegla schmitti* is relatively abundant in epigean streams in the Upper Ribeira Basin, southeastern Brazil and has a few records in caves. In this study, we questioned if those individuals would be an accidental presence or could be populations already established in cave streams? In this case, would morphological variations be associated with superficial and subterranean stream reaches? In this exploratory survey we tried to answer these questions by assessing the shape variation of cephalothorax using landmark-based geometric morphometrics. Different shapes were found for both environments, evidencing a successful hypogean colonization. The area effect was discarded as being the unique factor responsible for the divergence, therefore, for the first time in the literature, here we report morphological modifications associated with subterranean colonization in troglophiles. We also conceived and discussed some hypotheses about the colonization.

Key words: caves; morphometrics; subterranean colonization; troglophiles

INTRODUCTION

The species *Aegla schmitti* Hobbs III, 1979 is widely distributed in the Upper Ribeira Valley karstic area, southeastern Brazil (Rocha and Bueno 2004) and has been recorded in epigean and rarely recorded in hypogean stream reaches. In this last case, it was reported in a non linear pattern of distribution in Temimina I and II, Santana, Calcário Branco and Água Suja caves, which are separated by insoluble rocks in the studied area (see Moracchioli 1994; Bond-Buckup and Buckup 1994 and Rocha and Bueno 2004 for occurrence and geological map available at Sallun and Sallun Filho 2009).

Although not yet classified as a facultative cavernicole (= troglophile *sensu* Barr 1968) depigmented specimens of *Aegla schmitti* were mentioned by Moracchioli (1994) in a subterranean environment. Laboratory experiments resulted in gradual depigmentation in individuals maintained in a regime of darkness and without the addition of carotenoids in their diet, in a period of about a year. The absence of light and low content of carotenoids (available only in vegetal debris) is similar to the conditions found in cave streams. Even though long term studies are lacking, it is believed that this species could be capable of establishing viable populations in subterranean as well as superficial stream reaches (Moracchioli *Op. cit.*).

When a species occurs in both environments (as *A. schmitti*) it is not always clear to what is its degree of dependence on the subterranean environment, as well as it is not clear the degree of connectivity that is achieved among those subpopulations. As a matter of fact, different degrees of specialization to subterranean realm can even be observed in subpopulations, from the same biological species, as a consequence of the connectivity between them and the adaptability to each specific habitat condition (Wilkens 1988). In this regard, authors as Trajano et al (2009), Trajano (2012) and Poulson (2010) have already applied the concept of source-sink metapopulation (*sensu* Harrisson 1991) to subterranean populations.

In his classic book "On Growth and Form", Thompson (1917) discussed how morphology is related to mechanical and physiological efficiency in live organisms thus resulting ultimately from natural selection. In subterranean environments, not only the selective regimen (or even the relaxation of the selection), but the stochastic factors, besides geomorphology and historic factors, also play a fundamental role. Indeed, the colonization process itself subjects the subterranean fauna to genetic drift and founder effect (see founder principle, Mayr 1942) by initially reducing the genetic variability, while geomorphology influences the population connectivity (see Barr 1967. Wilkens 1988; 2010).

In fact, character states associated with isolation in subterranean environment often show up convergently in the most diverse taxonomic groups (Wilkens 2010). Morphologically, several troglobites have a more slender and elongated body and appendages than the closely related epigean species (Barr 1968; Culver 1982; Christiansen 2005). Such elongation of appendages has been explained as being sensory compensation (*e.g.* Mejía-Ortiz et al 2006), while the adaptive value of body elongation and slenderness seems to be associated with specific

Received 29 August 2012; Accepted 15 December 2012

habits and/or habitats (Barr 1968; Christiansen 2005; Trontelj et al. 2012).

Herein, we discuss the morphology of *Aegla schmitti* with an ecological evolutionary approach, searching for morphological modifications that could be attributed to life in subterranean environments, associated or not to subterranean selective regimes.

The chosen method was Landmarks-based geometric morphometrics, which consists of shape analysis by means of anatomical reference points, after eliminating effects of non-shape variation (position, orientation and scale of specimens) (Adams 1999; Giri and Loy 2008).

This exploratory technique has been successfully applied in studies with subterranean organisms like Reis et al (2006), in epigean and subterranean populations of armoured catfishes *Ancistrus cryptophthalmus* (Reis, 1987) and subterranean *Rhamdiopsis krugi* Bockmann and Castro 2010 (B. Rantin, in prep.). Both studies associated population divergences to isolation patterns between fish populations occurring in different cave systems (*A. cryptophthalmus*) or different limestone lenses (*R. krugi*).

Considering this, our purpose is to discuss two issues related to cave colonization of *A. schmitti*, by means of an exploratory analysis of shape: would those individuals be accidental or are populations already established in cave streams? In this case, would shape variations be associated to superficial and subterranean stream reaches? We also propose and discuss some colonization hypotheses.

METHODS

A. schmitti specimens were collected between March 2009 and August 2010, in subterranean and epigean stream reaches located at Parque Estadual Turístico do Alto Ribeira (PETAR) karstic area, São Paulo State, southeastern Brazil (Fig. 1).

Despite several field surveys in the area, including all caves with records of occurrence, individuals were captured only in Santana Cave (24°31′51″S and 48°42′06″W), and Betari River (24°31′14″S and 48°41′43″W, in a stream reach immediately downstream from outflow of the resurgence of Santana Cave river).

The collected specimens were deposited in the scientific collection of Laboratório de Estudos Subterrâneos, Universidade Federal de São Carlos – UFSCar (Numbers 1038 and 1039). Loans of individuals were also obtained from Museu de Zoologia from Universidade de São Paulo (MZUSP) and Laboratório de Estudos de Eglídeos from Universidade de São Paulo (LEEUSP) scientific collections.

Only adult specimens were used for geometric morphometrics, in a total of 24 males and two females from Santana Cave; 84 males and 50 females from Betari River; two males and two females from Temimina II Cave and three females from Água Suja Cave. The last two localities were not analyzed because of the small size of the sample. Although it seems to be a small sample to undertake the geometric morphometrics, it is more than the minimum adequate to the number of coordinates (see Monteiro and Reis 1999). Also, after several collections we could not catch more individuals which can indicate that these populations are threatened. Therefore, more samplings are not justified.

The specimens were oriented by the dorsal region and, then, photographed using a digital camera (SONY-H7). The images were converted to TPS format with TPSUtil version 1.44 (Rohlf 2009) and the homologous coordinates were obtained using TPSDig 2 version 2.12 (Rohlf 2008 a). A total of 11 landmarks were digitized on each specimen (Fig. 2) and pixels were transformed in millimeters by means of a scale inserted in the picture. Also, the repeatability of the landmarks was checked by means of Analysis of Variance (ANOVA) in Generalized Procrustes Analysis (GPA, Bookstein 1986) aligned coordinates of 30 images, marked three times.

Subsequently, each configuration was submitted to symmetrization, as described in Mardia et al (2000), to reduce the influence of variations related to asymmetry and avoid inflation of degrees of freedom. It was done creating a reflected configuration, by multiplying the x coordinate of the landmarks by – 1 and then calculating a consensus configuration between original and reflected landmarks. This procedure was done in R version 2.11 (R Development Core Team 2009). All posterior analyses were undertaken with transformed coordinates.

We chose GPA (Bookstein 1986; Adams et al 2004) as the method of superimposition to remove non-shape variations, computed by TPSRelw version 1.46 (Rohlf 2008 b). In the same software we performed a Relative Warps Analysis. To have all landmarks in the same scale, the alpha selected was equal to zero, most suitable for exploratory investigations (Rohlf 1993).

For testing whether the chosen landmarks would be influenced by sexual dimorphism, a Non-parametric Multivariate Analysis of Variance (NPMANOVA), using Euclidean distance (Anderson 2001) was applied in the relative warps scores matrix (S' matrix) between males and females, only in the first nine principal components different to zero, to remove redundant information.

Since there was influence from sexual dimorphism, only males were tested for shape differences between Santana Cave and Betari River. Thus, a Non-parametric Multivariate Analysis of Variance (NPMANOVA), using Euclidean distance (Anderson 2001) was applied in the relative warps scores matrix (S' matrix) between Santana Cave and Betari River, in the first nine principal components different from zero.

All statistical analyses were computed using Past version 2.01 (Hammer et al 2001). Also, thin-plate splines analysis was done using Morpheus beta version (Slice 2002), using consensus configurations of males from Betari River and Santana Cave.

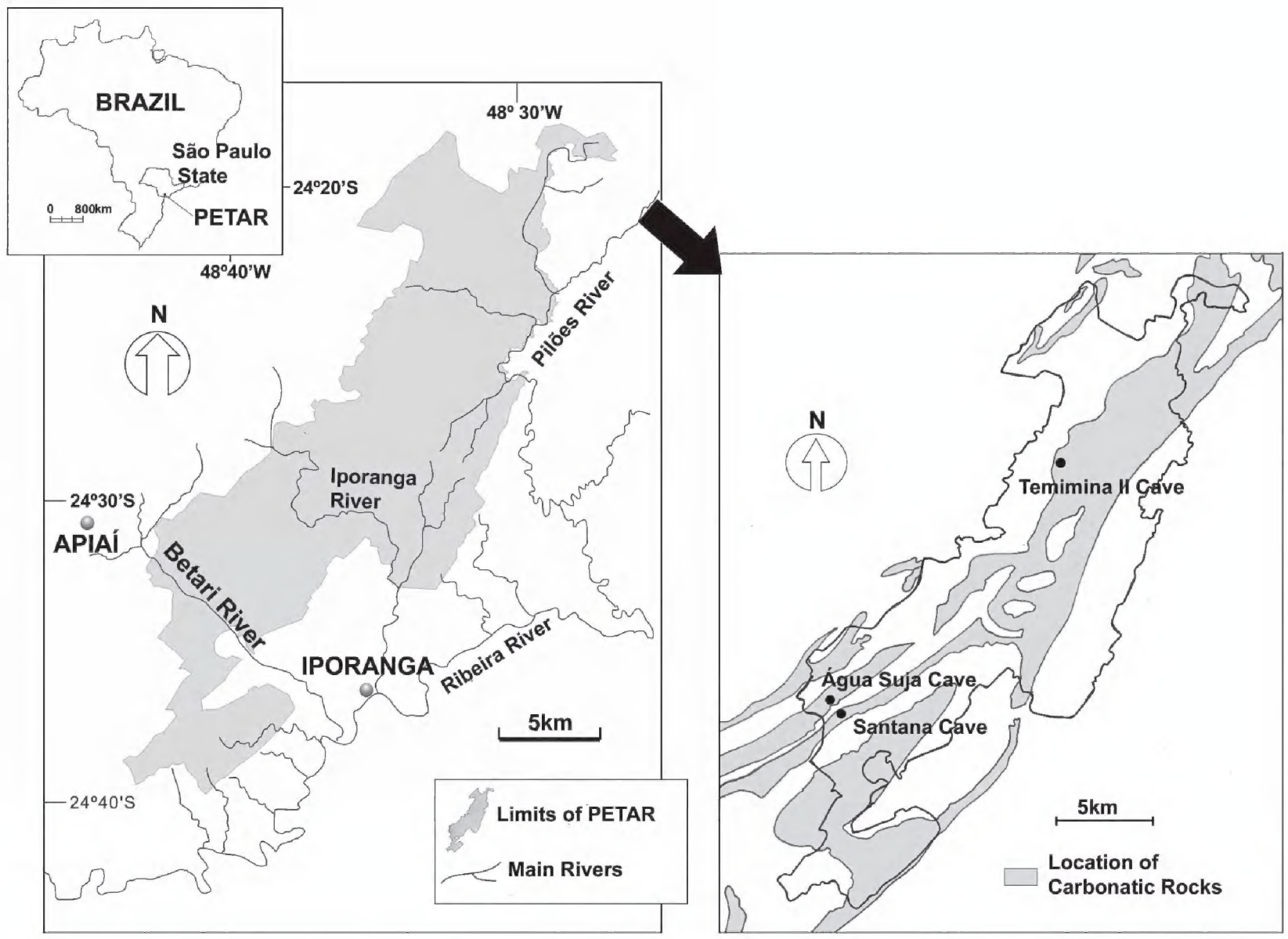

Fig. 1 - Limits of Parque Estadual Turístico do Alto Ribeira (PETAR) and location of limestone and caves in the study area. Modified from Karmann and Ferrari (2002).

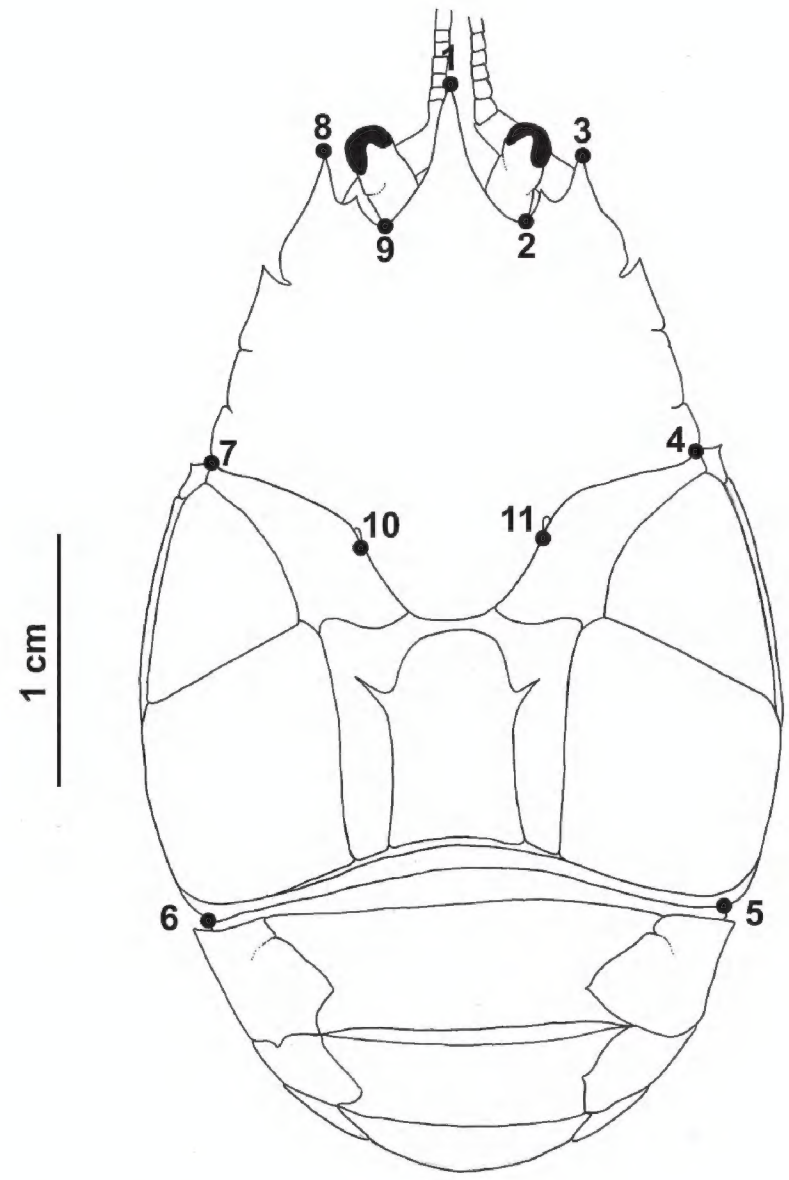

Fig. 2 - Dorsal view of *Aegla schmitti* showing the landmarks used in geometric morphometric analyses. 1 tip of the rostrum; 2 and 9 outer basis of eyestalk; 3 and 8 tip of anterolateral spine; 4 and 7 precervical width; 5 and 6 cephalothorax posterior region (marked where abdomen begins); 10 and 11 cervical groove apodeme.

RESULTS

The selected landmarks were repeatable and suitable for subsequent analyses (F=2.738E-10; p=1). Also, as they were influenced by sexual dimorphism, males and females were not grouped in later statistical analyses (F= 66.67 e p<0.0001).

There was a continuous and statistically significant morphological divergence between individuals from Santana Cave and Betari river (F=4.847; p=0.003). Moreover, though individuals from Betari river have been obtained next to the Santana Cave resurgence, they were more similar to the river specimens deposited in scientific collections (MZUSP and LEEUSP) (Fig. 3). The first two relative warps calculated between males from cave and river, with α=0 and uniform component included added up a total of 67.49% of the variance (first relative warp: 48.50%; second relative warp: 18.98%).

The landmarks responsible for the greatest variation on the first principal component were those related to eyestalk position and anterolateral spines (corresponding to numbers 2, 3, 8 and 9). Cave individuals had, as a rule, narrower cephalothorax and rostrum and a pre-cervical area more elongated than the epigean individuals, as illustrated by thin-plate splines diagrams (Fig. 4).

DISCUSSION

The epigean individuals sampled for this study, even being from a site next to the cave, showed greater similarities with the ones from the river, obtained from scientific collections (thus coming from different stream reaches and different time of collection). In fact, our results showed that not only the individuals from the river and from the caves were different, but also showed that a reduced gene flow between the extremes of distribution, as it was proposed by Vanzolini (1992) as being an Area Effect phenomenon, is not the unique factor influencing shape variation.

As demonstrated by Moracchioli (1994) in laboratory experiments, depigmentation in *A. schmitti* occurred in a period of about one year under dark conditions and low supplement of carotenoids in diet. As a consequence, the individuals of *A. schmitti* showing various degrees of depigmentation she found in subterranean stream reaches would be living for a relative period of time in this environment, and the depigmentation possibly occurred since in decapods it is associated with carotenoid availability on feeding (Maguire Jr. 1961; Wolfe and Cornwell 1964), which is often scarce in caves. Such facts, besides other records of this species in caves (Bond-Buckup and Buckup 1994; Rocha and Bueno 2004 and ours) showed that

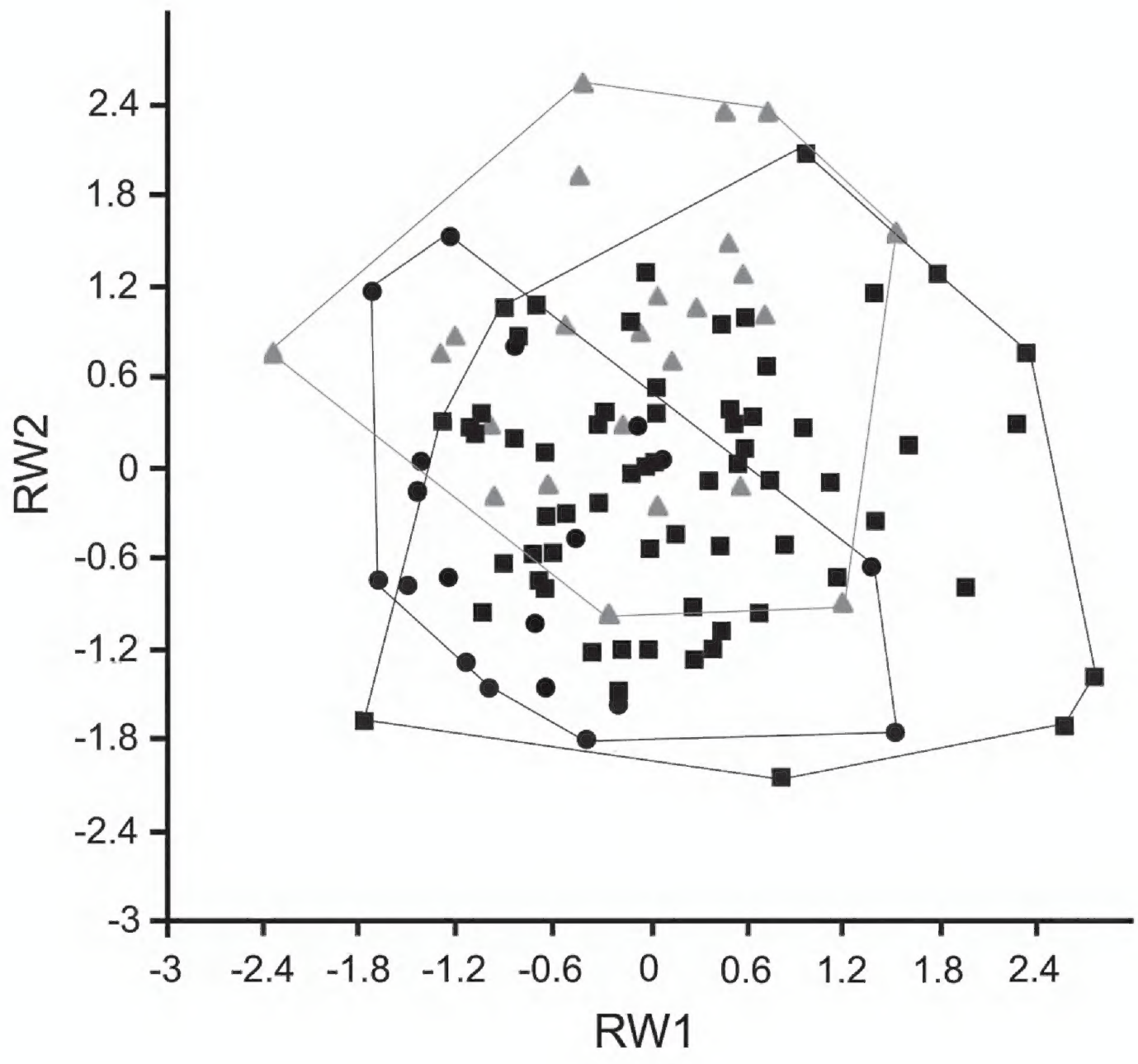

Fig. 3 - Relative Warps for values of α=0 of *A. schmitti* males from Betari River sampled specimens (circles), Betari River from scientific collections (squares) and from Santana Cave (triangles). RW1 = Relative warp 1 e RW2 = Relative warp 2.

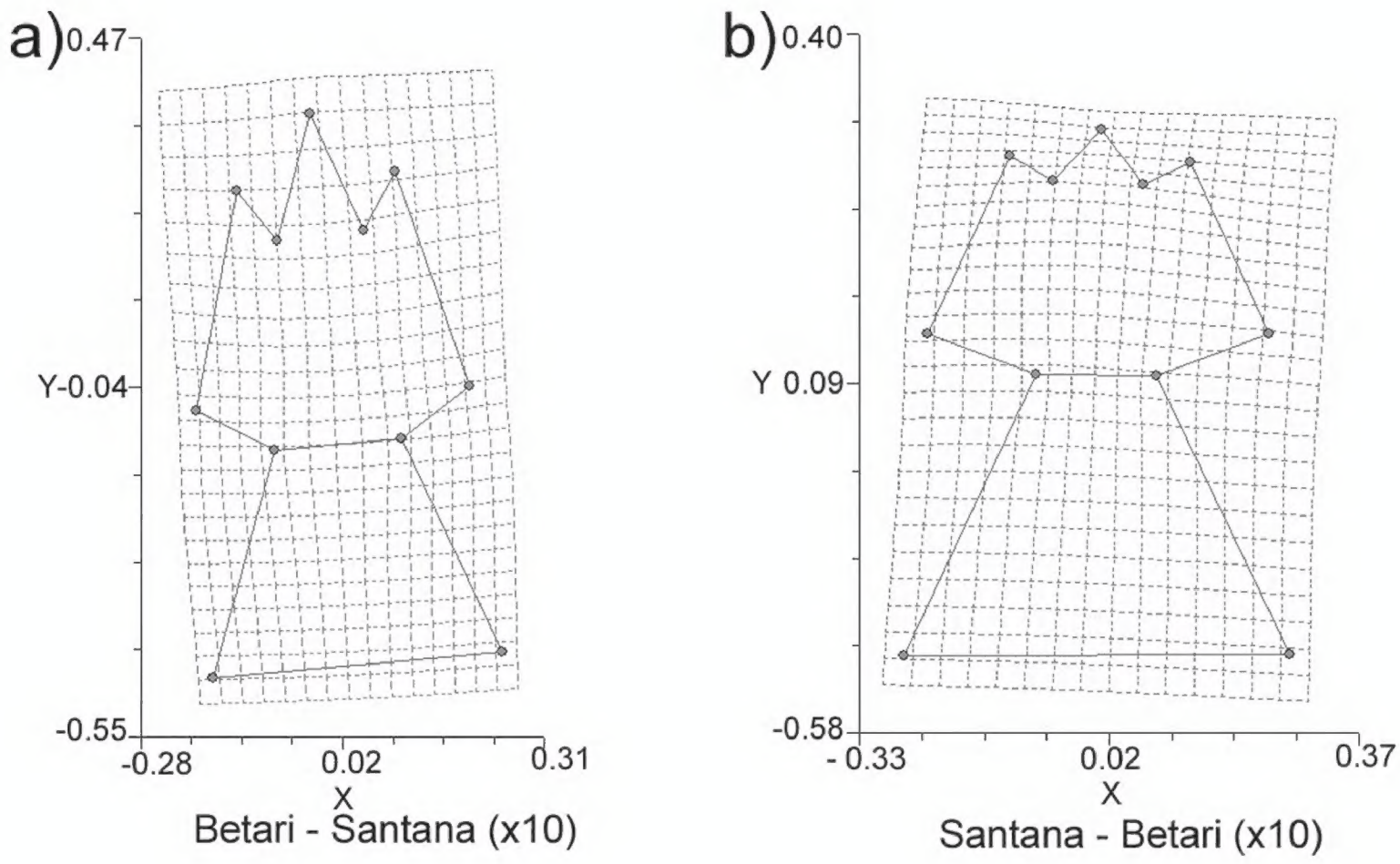

Fig. 4 -Thin plate splines diagrams showing deformations of a reference in a target configuration. (a) Betari River deformed in Santana Cave; (b) Santana Cave in Betari River. Results magnified 10 times for visualization of shape differences.

A. schmitti at least can establish and survive for a long period in this environment.

Nevertheless, as we said in the introduction, the records of *A. schmitti* in caves (Moracchioli 1994; Bond-Buckup and Buckup 1994; Rocha and Bueno 2004) show up in a non linear pattern, with the only exception of Temimina I and II caves, located very closely to each other in the same limestone outcrop. This pattern evidences the absence of a subterranean dispersion for this species (Barr 1967). So, although being capable of survival for a long period in subterranean stream reaches, the pattern demonstrates that the source population lies in epigean river.

Our results showed a continuous pattern of morphological divergence (Relative Warps Analysis, Fig. 3) which is consistent with a gene flow limited to occasional exchanges. Considering the distribution, the morphological divergence, and the fact that well established populations in past were not found in the present time (*e.g.* Temimina II and Água Suja Caves), we hypothesize that *A. schmitti* follows a source-sink metapopulation dynamics in the study area (*sensu* Harrisson 1991). Therefore, as the subterranean stream reaches are more restrictive to the establishment and reproduction of the species, the river population would be a source of new genes by means of migratory individuals, maintaining the peripheral population of Santana Cave as well as the other subterranean sink populations which were not found in the moment we sampled.

Sink populations correspond to groups of individuals stranded in habitats which are less than suitable. When the concept of source-sink populations is applied to the usual ecological-evolutionary classification of subterranean organisms, only the source populations are considered (Trajano 2012). According to this new definition, *A. schmitti* is classified as a trogloxene (source populations in epigean river). Otherwise, using the traditional classification (populations established and reproducing in both environments), the status of troglophile should be attributed.

Historical factors could also have influenced the observed morphological pattern. A set of geological and paleontological evidences (Ab'Saber 1977; Ledru 1993; Karmann 1994; Cruz et al 2005) shows that the study area was under Pleistocene climatic fluctuations as well as successive drainage alterations. Drier periods may have reduced streams levels, promoting successive scenarios of coalescence and isolation of Ribeira Valley waters (evidences from the troglobitic fish *Pimelodella kronei* (Ribeiro 1907), *sensu* Trajano and Britski 1992). Therefore, the aquatic environment fragmentation, associated with the isolation of troglophilic populations in subterranean environment, would enable the evolutionary processes to act, accumulating genetic differences during isolation and later introgressing with epigean population (Trajano 1995).

As *A. schmitti* is capable of establishing itself in subterranean environment, it must be investigated if and how climatic fluctuations (causing drainage alterations) would influence the genetic frequencies in these populations. Xu et al (2009), in a molecular study with *Aegla alacalufi* Jara and Lopez 1981 from Chile, reported divergences between populations supposedly under Pleistocene climatic fluctuations and those in refuge areas. In *Sclerobunus*, Banks 1893, a genus of Opiliones, it seems that the evolution of weakly troglomorphic forms (*i.e.*, with slightly reduced pigmentation, slightly elongated

appendages and no reduction in eye mound) is consistent with Pleistocene effects model (Derkarabetian et al 2010). According to Barnosky (2005) and Xu et al (2009), Quaternary climatic changes may have promoted a genetic change at population level. It is expected that anagenetic evolution in caves is accelerated in relation to that observed in epigean refuges, once subterranean ecosystems, depending on allochthonous food intake, may lose a great deal of diversity due to drastic food shortage (Trajano 1995).

Associated with partial reproductive isolation, a heritable higher capability to express specific responses to the environmental conditions can be expected to be favoured by natural selection (Stearns 1983; Romero and Green 2005). Experimental studies with tetracharacin fishes *Astyanax fasciatus* (Cuvier 1819) (Romero 2002) and epigean crayfishes *Cambarus tenebrosus* Hay 1902 (Cooper et al 2001) demonstrated that some troglomorphic forms differ from epigean ancestors not only genetically but, at least partially, also in environmentally induced features (Romero 2002; Romero and Green 2005).

The Aeglidae exhibits high intraspecific variation, which suggests they are capable of responding to different environmental conditions (see Giri and Collins 2004; Giri and Loy 2008). It is expected that in many caves, subjected to pronounced seasonality related to rainy cycles (Bichuette and Trajano 2003), plastic genotypes are selected, as environmentally induced phenotypes have higher probability to adapt to changes in environmental conditions than genetically fixed ones (Whiteman 1994; Stearns 1983; Romero and Green 2005).

Furthermore, genetic adaptation to specific habitats would favor those individuals with different morphological and physiological features between two environments, the ecotypes (Romero and Green 2005). Giri and Loy (2008) compared *Aegla neuquensis* Schmitt 1942 from lakes and rivers and reported the existence of ecotypes associated to both. Also, the cephalothorax shapes of river individuals were more variable than in the lake ones, which are features possibly associated to environmental conditions, predators and population connectivity.

The slenderness of cephalothorax and rostrum and, also, elongation of the pre-cervical area observed in the cave individuals from our study is similar to the morphological modifications already found in troglobitic (= obligate) cave fauna when compared to closely related epigean species. This trend towards body elongation and slenderness was observed in different areas and lineages of troglobites, evidencing that these traits are homoplasic cave-dependent features under adaptive control. Some authors associated these traits with habitat conditions and life habits, as an adaptation to squeezing through narrow crevices (Barr 1968; Hobbs 2000; Christiansen 2005; Trontelj et al 2012). This hypothesis is not applicable to our case, as *A. schmitti* occurs in a base-level subterranean stream reach thus not corroborating adaptation to narrow spaces. Nevertheless, the above mentioned trend is somehow convergently similar to traits found in cave obligate fauna and, therefore, it seems to be associated to subterranean colonization whether resulting from subterranean selective regime, ecotypes selection or phenotypic plasticity.

Small populations, mainly inside caves, can lose alleles by genetic drift, if gene flow with surrounding populations does not occur compensating this loss (Barr 1967). There is an increased preoccupation about *Aegla* in the study area as their natural habitats are being impacted (Moraes and Molander 2004; Cotta et al 2006) and a drastic population reduction in all troglobites was already observed (K. Maia, in press.). Our unsuccessful collections, not observed in previous studies (Moracchioli 1994; Bond-Buckup and Buckup 1994; Rocha and Bueno 2004) showed that *A. schmitti* could also be threatened.

In summary, our results indicated when mainly associated with epigean stream reaches, *A. schmitti* is not only capable of colonizing and establishing itself in the subterranean environment, but also that the morphological differences are associated with subterranean colonization. Further studies are needed to elucidate the ecological-evolutionary processes lying behind the apparent morphological convergence with troglobites. Also, due the partial isolation, effective populational size studies would be useful to evaluate the viability of the Santana Cave population and propose future conservation policies.

ACKNOWLEDGEMENTS

The authors would like to thank their colleagues from Laboratório de Estudos Subterrâneos by field assistance (T. L. C. Scatolini, D. Monteiro-Neto, J. E. Gallão and F. F. Franco). We also thank Drª Eleonora Trajano (IB/USP), Dr. Sergio L. S. Bueno (IB/USP) and Dr. Marcos Tavares (MZUSP), for granting the access to the scientific collections. The statistical analyses were performed with the help of Dr. Marco Batalha (UFSCar) and the shape analysis was oriented by Drª Érika Hingst Zaher and Fábio de Andrade Machado. We also thank Dr. Ivo Karmann and Dr. Carlos Grohmann (IGC/USP), for the loan of maps. This work was accomplished with financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), by means of Master's degree scholarship awarded to the first author (#13.6052/2009-6). The samplings were performed according to Brazilian State and Federal laws concerning wild animals (ICMBIO # 19.05l-1).

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